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Interaction of Normal and Glycated Human Haemoglobin with Erythrocyte Membranes from Normal and Diabetic Individuals

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Summary: The interaction between normal or glycated haemoglobin and the cytoplasmic surface of human erythrocyte ghost membranes from normal and diabetic individuals was studied at low pH and low ionic strength. Haemoglobin binding to the membrane was monitored by quenching of a fluorescent probe, 12-(9-anthroyl) stearic acid, embedded in the membrane. The quenching occurs by energy transfer from the probe to the membrane-bound haemoglobin molecules. It was found that both glycated and non-glycated haemoglobin bind with higher affinity to membranes from diabetics than to control erythrocyte ghosts. The binding of glycated haemoglobin is significantly less than that of normal haemoglobin to red blood cell membranes from both normal and diabetic individuals.

Introduction

Non-enzymatic glycosylation alters the physical and chemical properties of proteins. It has been reported that glycation changes the conformation and function of human serum albumin (1), inhibits the binding of fibronectin to matrix components (2), leads to partial unfolding of α-crystallin (3), decreases binding of sulphoxazole to serum albumin (4) and alters the ability of haemoglobin to bind oxygen and glycerate 2,3-bisphosphate (5). The levels of glycation of most proteins are elevated in diabetes mellitus. This phenomenon seems to be especially important for proteins with slow turnover rates such as lens crystallins (6), collagen (7), and the blood proteins: albumin (8), haemoglobin (9) and erythrocyte membrane proteins (10, 11).

Several studies have been reported on the binding of haemoglobin to erythrocyte membranes. It has been shown that haemoglobin binds to the cytoplasmic surface of this membrane, specifically to the cytoplasmic domain of band 3 protein. This binding is reversible and mainly electrostatic in nature (12–18). Oxy-, deoxy-, and sickle cell haemoglobins were found to bind to the band 3 with different affinities (17, 19, 20). In view of the above findings it seemed of particular interest to study the association of normal and glycated haemoglobins with erythrocyte membranes from normal and diabetic individuals. This was achieved by measurements of resonance energy transfer between a fluorescent probe inserted into the membrane and the haem of membrane-bound haemoglobin under conditions of low pH, low ionic strength and low concentrations of haemoglobin. The method has been described in detail by *Shaklai* et al. (12).

Materials and Methods

Chemicals were of analytical grade obtained from POCH (Gliwice, Poland) and Sigma Ltd. (glucose, fluorescent label 12-(9-anthroyl) stearic acid, human haemoglobin). Haemoglobin was used without further purification and considered as non-glycated Hb.

Glycation of haemoglobin

Human haemoglobin (5 g/l) was incubated in a 1.67 mol/l solution of glucose in 10 mmol/l sodium phosphate buffer, pH 8.0 with 0.2 g/l sodium azide at 37 °C for 98 hours. At the same time a control sample was run in which haemoglobin was incubated under the same conditions, but without glucose in the incubation medium. At the end of the incubation, the haemoglobin solutions (1.85 g/l Hb) were dialysed against distilled water, and then against 5 mmol/l phosphate buffer, pH

6.0. These solutions were used for fluorescence quenching measurements. Haemoglobin was determined by the method of *Drabkin* (21). As the rate of formation of the *Amadori* product for haemoglobin is about 4–5 times lower compared to human serum albumin, the incubation of haemoglobin with glucose should result in a glycated haemoglobin formation of 0.05% per day and mmol glucose (22, 23). Haemoglobin obtained under these experimental conditions is here-after called "glycated haemoglobin".

Preparation of labelled ghosts

Blood samples from 8 type I diabetic patients were obtained from the Diabetological Clinic of the Medical Academy of Lodz. Samples from 8 healthy control patients were from the Central Blood Bank of Lodz. Red blood cell ghosts, free of haemoglobin, were obtained exactly as previously described (12). Briefly, erythrocytes were washed 4 times with phosphate-buffered saline (0.15 mol/l NaCl in 5 mmol/l potassium phosphate, pH 8.0). Haemolysis was performed at a 1:40 volume ratio of packed erythrocytes to 5 mmol/l phosphate buffer at pH 8.0. Next, ghosts were washed 5–6 times with this buffer until the supernatant was free of haemoglobin. The optical absorbances of ghost suspensions were measured at 415 nm. Ghosts which contained less than 0.5% of the haemoglobin which was added during fluorescence measurements were considered to be free of haemoglobin. The unsealed ghosts were equilibrated with 50 mmol/l NaCl at pH 8.0 to release membrane-bound aldolase and glyceraldehyde-3-phosphate dehydrogenase (24), and the salt was then replaced by 5 mmol/l phosphate buffer, pH 8.0 by dialysis. Ghosts were labelled with the negatively charged fluorophore 12-(9-anthroyl) stearic acid in a 1:200 probe to membrane lipids weight ratio. After 1 hour incubation, the labelled membranes were washed with 5 mmol/l phosphate buffer, pH 8.0. Finally, ghosts were suspended in 5 mmol/l phosphate buffer at pH 6.0 at a ghost concentration of 6×10^6/l. For each sample, fluorescence quenching by normal and glycated haemoglobin was determined.

Fluorescence measurements

All fluorescence measurements were performed at 25 °C with a JY-3 spectrofluorimeter Jobin-Yvon, Longjumeau, France. Fluorescence quenching was achieved by the addition of small aliquots (1 µl) of Hb or glycated Hb solution to the 3 ml of ghost suspensions. As a result of haemoglobin binding to the membrane, the acceptor (haem) and the donor (12-(9-anthroyl) stearic acid) groups are brought sufficiently close for energy transfer to occur, resulting in a reduced fluorescence emission from 12-(9-anthroyl) stearic acid. The excitation wavelength was 360 nm and the emission fluorescence intensity was monitored at 480 nm to diminish scattering from excitation light and reabsorbance of the fluorescent light by haemoglobin at 415 nm.

Binding analysis

Assuming that the binding of each haemoglobin molecule causes the same quenching of the fluorescence intensity, it was possible to determine the binding constant, K_a, as described by *Lehrer & Fasman* (25):

$$K_a = \frac{\beta}{1-\beta} \cdot \frac{1}{[L_f]} \qquad \text{(Eq. 1)}$$

where $\beta = \frac{F - F_o}{F_L - F_o}$, and $[L_f] = [L] - \beta[C]$; F_o, F_L, and F are the fluorescence intensities of unliganded binding sites, fully liganded binding sites, and of the experimental mixture, respectively; $[L_f]$ and [L] are the concentrations of free haemoglobin and total haemoglobin present, respectively, and [C] is the concentration of binding sites in mol per litre. The value of the binding constant was obtained by extrapolation of a plot of $1/F - F_o$ vs $1/[L]$ to $1/[L] = 0$ to obtain $1/F_L - F_o$. The binding constant, K_a, was calculated from the slope of a plot of $[L_f]$ vs $\beta/1-\beta$ (26).

Results

The binding of haemoglobin to erythrocyte ghost membranes was examined by fluorimetric titration. The emission spectra of membrane-bound 12-(9-anthroyl) stearic acid were typical for this fluorescent probe (12), with the maximum at 440 nm. The addition of haemoglobin to 12-(9-anthroyl) stearic acid-labelled erythrocyte membrane suspensions resulted in a quenching of the fluorescence intensity but it did not change the shape of the spectrum, or the position of the maximum. This suggests that the decrease in the fluorescence quantum yield was caused by energy transfer from 12-(9-anthroyl) stearic acid to haemoglobin, which has an absorbance maximum at 415 nm. The quenching experiments were performed with erythrocyte ghosts from both non-diabetic and diabetic individuals in 8 independent experiments. Two solutions of haemoglobin were used as described in Materials and Methods, one considered to contain non-glycated, the other glycated haemoglobin.

The plots of the data obtained according to Equation 1 are shown in figure 1. Each point of the plots represents the mean value taken from 8 measure-

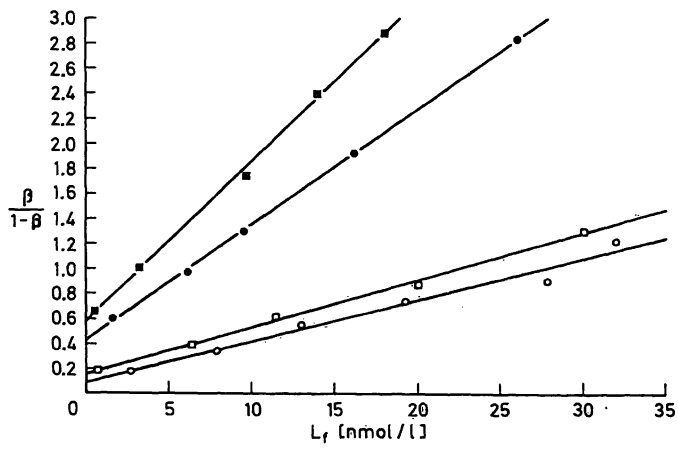
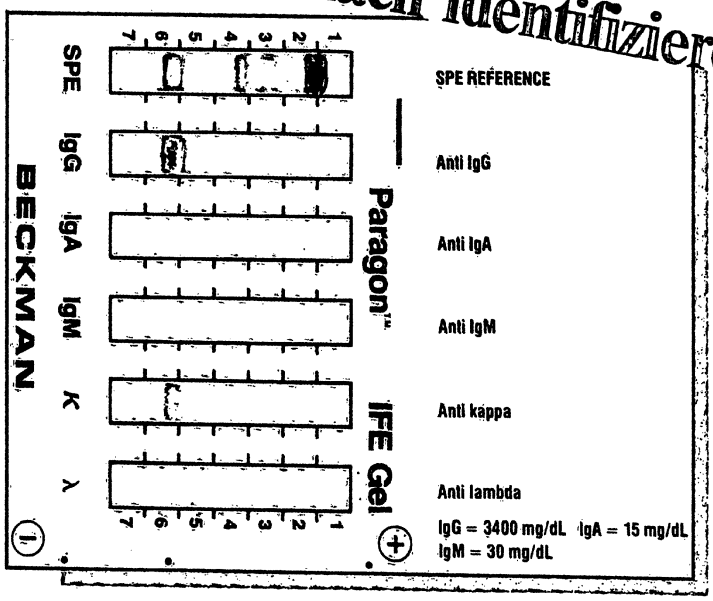


Fig. 1. Fluorimetric titration of human erythrocyte ghosts from diabetic and non-diabetic individuals with glycated and non-glycated haemoglobin
For further explanations see Materials and Methods, section Binding analysis
- o membranes from non-diabetic individuals + glycated haemoglobin
- ● membranes from non-diabetic individuals + normal haemoglobin
- □ membranes from diabetic individuals + glycated haemoglobin
- ■ membranes from diabetic individuals + normal haemoglobin

Proteinanomalien schnell und einfach identifizieren . . .



mit der Beckman IFE Immunfixations-Elektrophorese.

Immunfixation (IFE) ist ein Verfahren, bei dem die Prinzipien der Proteinelektrophorese und der Immunpräzipitation kombiniert werden. IFE wird am häufigsten zur Identifizierung monoklonaler Immunglobuline eingesetzt und besticht durch ihre einfache Methodik.

Bei der Beckman IFE wird die Einzelprobe auf mehreren Positionen eines Agarosegels elektrophoretisch getrennt. Daran anschließend wird jede Trennung mit monospezifischen Antiseren gegen IgG, IgA, IgM sowie Kappa- und Lambda-Immunglobulinproteine bedeckt.

Eine ebenfalls auf dem Gel durchgeführte Referenztrennung zeigt die Wanderungsposition der speziellen Proteine in Relation zum Proteingesamtmuster des Serums. Das Gel wird dann 30 Minuten inkubiert. In dieser Zeit entwickeln sich die Immunkomplexe zwischen den spezifischen Antigenen und ihren entsprechenden Antikörpern. Im Anschluß an die Inkubation mit Antiseren wird das Gel mit Paragon-Blau zur Sichtbarmachung der Proteinbanden angefärbt.

Das in der Abbildung dargestellte Proteinmuster belegt eindrucksvoll die Eignung der IFE-Technik zur Identifizierung monoklonaler Proteine. Die spezifischen Antiseren gegen IgG und Kappa-Proteine zeigen deutlich den im Serumprotein-Elektrophorese-Muster auf der linken Seite des Gels sichtbaren, monoklonalen Peak als eine Zusammensetzung aus schweren IgG-Ketten und leichten Ketten vom Typ Kappa-Proteine.

Neben der Identifizierung von minimonoklonalen und biklonalen Proteinen wird die Immunfixation auch zum Nachweis von Mobilitätsveränderungen bestimmter Proteine und zur Erkennung der Antikörperklasse monoklonaler Proteine in der Cerebrospinalflüssigkeit von Patienten mit SSPE (subakute sklerosierende Panenzephalitis) und MS (Multiple Sklerose) eingesetzt.

Die Beckman IFE-Technik zur Identifizierung von Proteinen bietet entscheidende Vorteile gegenüber konventionellen Methoden:

1. Zeitersparnis
Die positive Identifikation der meisten monoklonalen Proteine kann nach Immunglobulin-Klassen und -Typen innerhalb von 2 ½ Stunden erfolgen.

2. Leichte und schnelle Interpretation
durch klare, scharf getrennte Banden.

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ments. Straight lines were fitted by the method of the least squares. From the slopes of these plots the binding constants, K_a, for each combination of ghosts and haemoglobins were calculated. Results are given in table 1. The double reciprocal plots of the percentage of fluorescence quenched (β) versus free haemoglobin concentration (L_f) are shown in figure 2. The fractions of fluorescence quenched at infinity ($1/L_f \rightarrow 0$) are different for glycated and non-glycated haemoglobin, and they also depend on the type of erythrocyte ghosts. The highest value for association is that of non-glycated Hb with ghosts from diabetics (85%). Results are summarized in table 2.

Tab. 1. Binding constants of glycated and non-glycated haemoglobin to erythrocyte ghost membranes from diabetic and non-diabetic individuals

Sample	$K_a \times 10^8$ [mol/l]$^{-1}$		
	Haemoglobin		Glycated haemoglobin
Control	0.91 ± 0.06	$p < 0.001$	0.32 ± 0.04
	$p < 0.05$		N.S.
Diabetes	1.27 ± 0.09	$p < 0.001$	0.35 ± 0.03

Results are expressed as mean ± SD, n = 8

Tab. 2. The fractions of fluorescence quenched at infinity ($L_f \rightarrow \infty$) by Hb and glycated haemoglobin for erythrocyte ghost membranes from normal and diabetic individuals

Sample	Fraction		
	Haemoglobin		Glycated haemoglobin
Control	0.82 ± 0.013	$p < 0.001$	0.70 ± 0.021
	N.S.		$p < 0.05$
	0.85 ± 0.009	$p < 0.01$	0.79 ± 0.017

Results are expressed as mean ± SD, n = 8

Discussion

Measurements of resonance energy transfer between a fluorescent probe inserted into the lipid bilayer of an erythrocyte membrane and the haem of membrane-bound haemoglobin provided values for the binding constants of red blood cell membranes from non-diabetics or diabetics to normal or glycated human haemoglobin. The binding studies were done at low concentrations of haemoglobin, low ionic strength, and low pH. Such conditions are considered to cause maximal binding of haemoglobin to the erythrocyte membrane (27, 28), but are far from being physiological. The value of K_a for Hb binding to non-diabetic membrane is in good agreement with the binding constant obtained by *Shaklai* et al. (12) using the same experimental conditions ($K_d = 0.85 \times 10^8$ (mol/l^{-1})). It was later suggested that at the concentrations of Hb required for fluorescence studies ($\sim 10^{-7}$ mol/l), oxyhaemoglobin is 97% dimeric, not tetrameric, and the new value for the binding constant was 0.53×10^7 (mmol/l)$^{-1}$ (24). Results given in table 1 and 2 show that non-glycated haemoglobin exhibits a higher affinity for membranes from diabetics as compared to control erythrocyte ghosts. On the other hand, the binding of glycated haemoglobin to red blood cell membranes from both normal and diabetic individuals is significantly impaired. However, because glycated haemoglobin is only a minor fraction of total haemoglobin, even in diabetic subjects, the increased binding of non-glycated haemoglobin to erythrocyte membranes from diabetics seems to be the predominant effect of haemoglobin binding in diabetic individuals. Additionally, our experience with the preparation of erythrocyte ghosts indicates that membranes from diabetics usually need more intensive washing to give haemoglobin-free white ghosts. It has been shown that the band 3 protein is a haemoglobin-binding site in the erythrocyte membrane (12–17). The amino-terminal 23-residue fragment of band 3, with 14 of its residues being negatively charged, is required for haemoglobin binding (18). This finding provided additional evidence that bind-

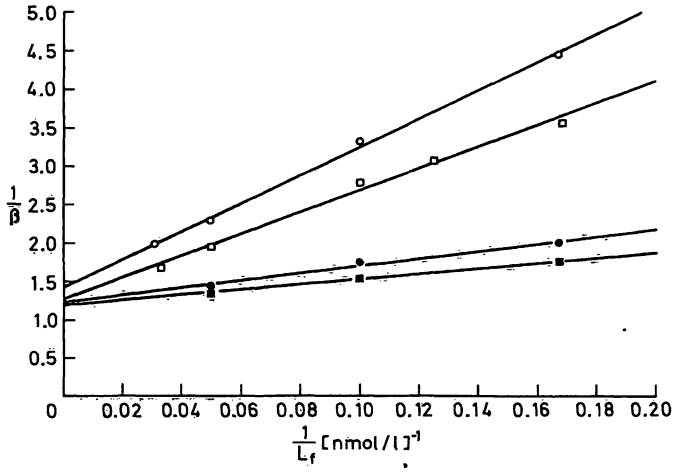

Fig. 2. Double reciprocal plots of the fraction of fluorescence intensity quenched versus free haemoglobin concentration.
For further explanations see Materials and Methods, section Binding analysis
- o membranes from non-diabetic individuals + glycated haemoglobin
- ● membranes from non-diabetic individuals + normal haemoglobin
- □ membranes from diabetic individuals + glycated haemoglobin
- ■ membranes from diabetic individuals + normal haemoglobin

ing of haemoglobin to the membrane is electrostatic in nature. It has been postulated that association depends also on the size of the positive charge on haemoglobin, since a direct relationship was observed between the positive net charge of different haemoglobin species and their affinities for erythrocyte membranes (29). Furthermore, it has been shown that the β chain of haemoglobin binds preferentially to the erythrocyte membrane (30). Thus the decrease of binding constant for glycated haemoglobin may be due to the fact that glycation of the N-terminal amino groups of the β chains of haemoglobin causes a decrease in the positive charge of the protein (31), thus diminishing the electrostatic interactions between the β chain of haemoglobin and the negatively charged region of the band 3 protein. At this stage of the experiments, it is difficult to offer a reason for the higher affinity of membranes of diabetics both for glycated and for non-glycated haemoglobin. One could postulate that the altered net charge of membrane proteins caused by increased glycation of membranes from diabetics (10, 11) is responsible for this effect. It was also estimated that approximately 50% of band 3 protein may normally interact with haemoglobin and that in pathological cases with the depletion of glycerate 2,3-bisphosphate, more haemoglobin can bind to band 3 since glycerate 2,3-bisphosphate competes with band 3 for deoxyhaemoglobin (17). Thus the lower glycerate 2,3-bisphosphate concentrations found in diabetes mellitus (33), should result in an increased pool of haemoglobin able to bind to the membrane. As a result there would be less free band 3 protein available for binding other cytosolic proteins, i. e. the glycolytic enzymes.

All these findings lead to the conclusion that under physiological conditions one can expect alterations in haemoglobin binding to the erythrocyte membrane in diabetes, although the biological significance of this association remains unclear. It was suggested (15) that haemoglobin molecules that interact with the membrane may act as perturbants to the dynamic structure of the cell membranes, affecting their structural and functional properties. This could have pathological consequences as was found for sickle red blood cells, where enhanced binding of haemoglobin was reported (32). However, further studies of haemoglobin-erythrocyte membrane interactions in diabetes under physiological conditions are needed to establish the existence of differences in these interactions under these conditions, and their possible pathological role.

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